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Cross-species analysis of thyroperoxidase inhibition by xenobiotics demonstrates conservation of response between pig and rat☆

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ABSTRACT

Thyroperoxidase (TPO), the enzyme that catalyzes the synthesis of thyroid hormone, is a known target for thyroid-disrupting chemicals. *In vivo* toxicological evidence supporting TPO-inhibition as one molecular-initiating event that leads to thyroid disruption is derived largely from rat models; however, a significant fraction of research on the inhibition of TPO by xenobiotics has been conducted using porcine TPO. The current work tested the hypothesis that porcine and rat thyroid microsomes exposed to TPO-inhibiting chemicals would demonstrate different responses in a guaiacol oxidation assay. A primary objective of this work is to establish the degree of concordance between rat and porcine TPO inhibition data. Microsomes were isolated from both rat and pig thyroid glands, and the guaiacol oxidation assay was performed for a training set of 12 chemicals, including previously reported TPO inhibitors, thyroid-disrupting chemicals thought to perturb other targets, and several previously untested chemicals, to determine the relative TPO inhibition responses across species. Concentration–response curves were derived for methimazole (MMI), dibutylphthalate (DBP), diethylhexylphthalate (DEHP), diethylphthalate (DEP), 3,5-dimethylpyrazole-1-methanol (DPM), iopanoic acid (IOA), 2-mercaptobenzothiazole (MBT), sodium perchlorate (PERC), p-nonylphenol (PNP), 4-propoxyphenol (4POP), 6-propylthiouracil (PTU), and triclosan (TCS). MMI, PTU, MBT, DPM, 4POP, and at extremely high concentrations, PERC, inhibited TPO activity. Results demonstrated a strong qualitative concordance of response between the two species. All chemicals that inhibited TPO in porcine microsomes also inhibited TPO in rat microsomes. Hill model-derived IC_{50} values revealed approximate 1.5- to 50-fold differences in relative potency to MMI between species for positive chemicals. DPM, MBT, 4POP, and PTU exhibited greater relative potency to MMI using rat TPO versus porcine TPO, but rank order potency for inhibition was similar for the other test chemicals, with: PTU > MBT > DPM > 4POP > PERC for rat TPO and MBT > PTU > DPM > 4POP > PERC for porcine TPO. These data support the extrapolation of porcine TPO data to potential thyroid-disrupting activity in rodent models to evaluate TPO-inhibiting chemicals.

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Abbreviations: 4POP, 4-propoxyphenol; CCP, complement control protein repeat domain; DEP, diethylphthalate; DEHP, diethylhexylphthalate; DBP, dibutylphthalate; DIT, diiodotyrosine; DPM, 3,5-dimethylpyrazole-1-methanol; EGF, epidermal growth factor; hTPO, human thyroperoxidase; IC_{50}, 50% inhibitory concentration; IOA, iopanoic acid; LCB, lower confidence bound; MBT, 2-mercaptobenzothiazole; MIT, monoiodotyrosine; MMI, methimazole; MPO, myeloperoxidase; NIS, sodium-iodide symporter; pTPO, porcine thyroperoxidase; PERC, sodium perchlorate; PNP, 4-*n*-nonylphenol; PTU, 6-propylthiouracil; rTPO, rat TPO; T3, triiodothyronine; T4, thyroxine; TCS, triclosan; Tg, thyroglobulin; TPO, thyroperoxidase; TSH, thyroid-stimulating hormone; UCB, upper confidence bound; unb, unbounded.

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1. Introduction

Thyroperoxidase (TPO) is a heme-containing apical membrane protein that acts as the enzymatic catalyst for the initial steps in thyroid hormone synthesis within the follicular lumen of thyrocytes. TPO catalyzes the oxidation of iodide, enables nonspecific iodination of tyrosyl residues of thyroglobulin (Tg) (Kessler et al., 2008), and couples iodotyrosyls to produce Tg-coupled monoiodotyrosine (MIT) and diiodotyrosine (DIT) (Divi and Doerge, 1994; Ruf and Carayon, 2006; Taurog et al., 1996). Hydrolysis of MITs and DITs from Tg results in the release of thyroid hormones, thyroxine (T4) and triiodothyronine (T3), from the gland into systemic circulation, with T4 representing the predominant product of this synthesis (Zoeller et al., 2007a). Inhibition of TPO activity is an accepted molecular-initiating event for a chemical-induced adverse outcome pathway of thyroid hormone disruption in rodent models (Crofton, 2008; DeVito et al., 1999; Doerge and Chang, 2002; Flippin et al., 2009; Hurley, 1998; Zoeller and Crofton, 2005). The causative link between TPO inhibition and depressed thyroid hormone concentrations has been well-established, as antihyperthyroid pharmaceuticals including methimazole (MMI) and 6-propylthiouracil (PTU) decrease systemic thyroid hormone concentrations in humans and pets with Graves' Disease (Emiliano et al., 2010; Trepanier, 2006). TPO inhibitors also reduce serum and/or tissue thyroid hormone concentrations in amphibian and avian species (Coady et al., 2010; Grommen et al., 2011; Rosebrough et al., 2006; Tietge et al., 2012). Thus, TPO inhibition is a relevant target for thyroid-disrupting chemicals across species, and exposure to TPO-inhibiting chemicals may pose human-relevant hazard, especially during development. The availability of T4 modulates fetal brain development, and even subclinical decreases in maternal T4 may culminate in irreversible, adverse neurological outcomes in children (Berbel et al., 2009; Kooistra et al., 2006; Li et al., 2010; Pop et al., 1999, 2003). Development and use of appropriate surrogate models is therefore important for determination of potential developmental neurotoxicants that act via TPO-inhibition.

A predominant model used to characterize potential TPO-inhibiting activity has been porcine TPO (pTPO), derived from thyroid follicles, microsomes, or partially purified protein fractions. Potassium cyanide, sodium azide, 3-amino-1,2,4-triazole (amitrole), thiouracil, 6-propylthiouracil (PTU), p-aminobenzoate, potassium thiocyanate, potassium perchlorate, sodium fluoride, and thiourea were all characterized as TPO-inhibitors approximately fifty years ago using partially purified pTPO and the guaiacol oxidation assay (Hosoya, 1963). The guaiacol oxidation assay for peroxidation activity utilizes H_2O_2 as a hydrogen-donor for TPO-mediated one electron oxidation of guaiacol to a yellowish-brown di-guaiacol product detected by spectrophotometric analysis (Chang and Doerge, 2000; Hosoya, 1963). Concern regarding dietary isoflavones, including genistein and daidzein, as potential goitrogens was also evaluated with pTPO preparations, with confirmation of TPO inhibition by these compounds in the absence of iodide (Divi et al., 1997). Ethylene thiourea, a common biotransformation product of ethylene bisdithiocarbamate pesticides (Doerge and Takazawa, 1990); *N*,*N*,*N*′,*N*′-tetramethylthiourea, a neoprene vulcanization agent (Freyberger and Ahr, 2006); resorcinols, produced in resin manufacturing (Divi and Doerge, 1994); sulfamethazine, an antimicrobial sulfa drug (Capen, 1998; Doerge and Decker, 1994); and leucomalachite green, an aquaculture pesticide (Doerge et al., 1998), were also identified as a TPO-inhibitors using pTPO. Cultured porcine follicle-derived TPO has demonstrated responsivity to MMI and PTU (Sugawara et al., 1999). While many studies demonstrate the utility of pTPO in assessing TPO activity in response to chemical exposure, there are no known *in vivo* studies of thyroid hormone status in pigs exposed to TPO-inhibiting chemicals. Instead, these studies are typically conducted in rats (Brucker-Davis, 1998; Chang and Doerge, 2000; Hood et al., 1999a; Hurley, 1998).

Many *in vivo* rat studies have previously reported decreased systemic concentrations of thyroid hormones following exposure to TPO-inhibiting chemicals, and this has often been further linked to adverse neurodevelopmental consequences subsequent to perinatal thyroid hormone insufficiency (Zoeller and Crofton, 2005). Often, the drugs MMI and PTU have been used as prototypical thyrotoxicants to demonstrate the relationship between thyroid hormone status and specific indicators of cytoarchitectural development of the brain, as well as behavioral alterations (Auso et al., 2004; Berbel et al., 2010; Lavado-Autric et al., 2003; Morte et al., 2010; Royland et al., 2008). Rat studies with PTU suggest that TPO inhibition, and a related decrease in thyroid hormones synthesis, results in a greater magnitude of effect on serum thyroid hormone and thyroid-stimulating hormone concentrations and thyroid histopathology when compared to hepatic microsomal enzyme inducers that disrupt thyroid hormones via up-regulation of thyroid hormone metabolism, like phenobarbital and pregnenolone-16α-carbonitrile (Hood et al., 1999b). A correlative relationship between exposure to TPO-inhibiting chemicals and decreased thyroid hormones *in vivo* has been well-illustrated with rodent models (Ahmed et al., 2012; Axelstad et al., 2008; Fegert et al., 2012; Gilbert, 2004; Gilbert and Sui, 2006; Goldey et al., 1995; Pathak et al., 2011), but typically the intermediary linkage, i.e. rat TPO (rTPO) inhibition assays, have not been conducted with rat thyroid tissues. An exception is genistein, for which comparative, confirmatory *in vivo* and *in vitro* evidence is available in rat models (Doerge and Sheehan, 2002). Recently, a comparative analysis of MMI exposure in rats, rat thyroid tissue, and human thyroid tissues suggested concordance of qualitative response between rats and humans, but an increased sensitivity to MMI in rats (Vickers et al., 2012).

As very few reports have directly compared rat and porcine microsomal TPO activity, and the preponderance of *in vivo* data to support chemical-induced inhibition of TPO has been derived from rat models, greater understanding of the cross-species concordance of TPO models for a training set of chemicals would increase the utility of both porcine and rTPO data for evaluation of potential TPO-inhibiting chemicals or mixtures. Herein we present an interspecies comparison of TPO inhibition with a guaiacol oxidation assay using a 12-chemical training set comprised of previously reported TPO inhibitors as well as chemicals expected to have no effect. We hypothesized that inhibition of rat and porcine TPO-catalyzed oxidation of guaiacol by xenobiotics would demonstrate differences across species models, justifying the use of both pTPO and rTPO as models for prediction of potential thyroid-disrupting effects in humans.

2. Materials and methods

2.1. Porcine thyroid microsome preparation

Porcine thyroid glands, approximately 10 g each, were procured from an abattoir (Duluth, MN), snap frozen on dry ice, and stored at −80 °C. The method of Chang and Doerge (2000) was adapted for preparing microsomes as described previously (Tietge et al., 2012). Five different microsome preparations derived from pig thyroid glands were used in this study. The mean of the protein concentrations of these five batches of microsomes, as determined by a Bradford protein assay, were 15.3 ± 1.35 mg/mL (mean ± SD).

2.2. Rat thyroid microsome preparation

Untreated male Long-Evans rats (68–72 days old) were obtained from Charles River Laboratories Inc., Raleigh, NC in groups of 60 and acclimated 1–7 days in an American Association for Accreditation of Laboratory Animal Care International (AALAC) approved animal facility. Rats were pair-housed in plastic hanging cages (45 cm × 24 cm × 20 cm), with heat sterilized pine shavings bedding (Northeastern Products Corp., Warrenton, NC). Colony rooms were maintained at 21 ± 2 °C with 50 ± 10% humidity on a photo-period of 12L:12D. Food (Purina Rodent Chow #5001,

Barnes Supply Co., Durham, NC) and water were provided *ad libitum*. The standard, soy-containing Purina Chow #5001 has been characterized previously as replete with iodine (unpublished data). Animals fed this chow have similar thyroidal iodine concentrations and serum thyroid hormone concentrations as animals fed a soy-free, casein-based diet (Gilbert, 2011; Gilbert et al., 2013; Paul et al., 2012; Zhou et al., 2002). Tap water (Durham, NC water) is filtered through sand, then activated charcoal, and finally re-chlorinated to 4–5 ppm Cl^- before use in the animal facility. All animal procedures are approved in advance by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory of the US EPA. Rats were decapitated and thyroid glands are removed, weighed (approximately 10 mg per lobe), frozen in liquid nitrogen, and stored at −80 °C until use. Rat thyroid microsomes pools were made from 50 to 60 pooled rat thyroids totaling around 1 gram of tissue. Thyroid microsomes were prepared similarly to porcine microsomes (Chang and Doerge, 2000). Briefly, frozen thyroid glands were homogenized in 2 mL of ice-cold buffer, containing 0.2 mM potassium phosphate, 200 mM sucrose, 1 mM EDTA, and 500 U/mL catalase, per 0.01 mg of thyroid tissue. This homogenate was then centrifuged at 29.4 × *g* for 10 min at 4 °C to remove larger debris, and then ultra-centrifuged at 151,515 × *g* for 60 min at 4 °C. The supernatant was discarded, and the pellet was resuspended in homogenization buffer (without catalase), with 0.25 mL of buffer per 0.01 g of thyroid tissue using a teflon-coated mortar and pestle. Glycerol (5%) was added to the final microsomal preparation, and aliquots were stored at −80 °C until thawed one-time for use. The mean ± SD of the protein concentrations of the three batches of microsomes used in this study, as determined by a Bradford protein assay, was 1.07 ± 0.13 mg/mL.

2.3. Chemicals

The 12 chemicals in this training set were chosen to contain active and inactive chemicals for TPO inhibition. Some were based on previous reports of TPO-inhibition (MMI, PTU, MBT, PNP), pilot data with the pTPO model (4POP, DPM, DEP, DEHP, DBP), and literature reports of chemicals that decrease thyroid hormones, but possibly via different molecular initiating events (PERC, TCS, and IOA). These chemicals were solubilized in dimethyl sulfoxide (DMSO), and used to expose both porcine and rat thyroid microsomes. The chemical names, abbreviations, chemical class, CAS#, source, and structure are presented in Table 1. A wide concentration range was tested for each chemical, using the limit of solubility as the determinant of the maximum concentration tested; solubility was confirmed visually and by nephelometry.

2.4. Guaiacol oxidation assays

This assay has been described previously for both rat and pig thyroid microsomes (Chang and Doerge, 2000; Tietge et al., 2012). All reactions were conducted in 96-well plates with a final reaction volume of 200 μL. Thyroid microsomes were incubated with 35 mM guaiacol with test chemical exposure at 37 °C for 30 s, 300 μM H_2O_2 in 100 mM potassium phosphate buffer (pH 7.4) was added to initiate the reaction, and the oxidation of guaiacol was monitored spectrophotometrically at 450 nm for 120 s. The kinetic reaction was monitored during the linear phase, i.e. the first 60 s, and the change in absorbance was recorded for analysis.

Before beginning experimentation with test chemicals, the porcine and rat microsomes were evaluated and optimized for activity. For the rat microsomes a minimum qualifying absorbance change was set at 0.08 when read for 60 s at 450 nm; this required approximately 50 μg of protein per reaction in this study. The change in absorbance for the three rat microsome preparations used in this study ranged from 0.13 to 0.22 at 60 s with 50 μg protein. For the porcine microsome preparations, a 10 μL volume maximized the absorbance change, so this volume was used in the assays and equates to approximately 150 μg of total protein per reaction. The maximal absorbance change among porcine microsome preparations ranged from 0.08 to 0.24 indicating a lower specific activity compared to those of the rat. Thus, the guaiacol oxidation reactions were conducted to meet the optimal assay performance by species.

2.5. Data analysis

The background absorbance prior to addition of H_2O_2 was subtracted from the change in the absorbance per minute, and then this value was normalized to the mg of protein. Three separate experiments ($n = 3$), using three different microsome batches, were performed for both pTPO and rTPO experiments using technical triplicates for each concentration in each experiment. The rTPO experiments were carried out in three experimental sets in which all 12 chemicals were tested, whereas the pTPO experiments were carried out with various subsets of chemicals. When each of these subsets of chemicals were run in the pTPO experiments, methimazole was run as a positive control chemical; therefore there are more methimazole curves for the pTPO experiments ($n = 14$) than the three experimental runs for each of the other 11 test chemicals. The data were normalized to the vehicle control activity (100%) within each experiment. In some cases the normalized results demonstrated responses greater than 100% at the lowest concentrations tested, likely owing to experimental error rather than biological activity. To correct for shifts in the baseline above 100% of vehicle control activity, all normalized data were divided by the mean response of the lowest concentration tested and multiplied by 100. This baseline correction enabled the use of a Hill model constrained from 0 to 100 to fit the data, and derivation of an IC_{50} value equivalent to 50% of vehicle control activity for all chemicals tested (R version 2.15.2, scripts available upon request). The bottom and top of the Hill fit were constrained to 0% and 100%, respectively, with the Hill slope constrained from −1 to −8. The fit curves were used to predict the 50% inhibitory concentration (IC_{50}) for positive chemicals, defined as 50% activity, as well as the lower and upper 95% confidence bounds around this IC_{50} value (Table 2). A positive response in the TPO assays was defined as a 20% or greater decrease in activity from the vehicle control. Percent relative potency to MMI, within species, was calculated as the composite experimental MMI IC_{50} divided by the test chemical IC_{50}, and multiplied by 100 (Table 2), and this percent relative potency was the basis for comparison across species.

2.6. Sequence alignments for cross-species comparison

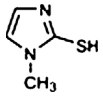
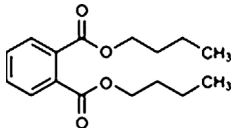
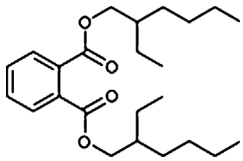
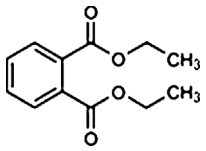
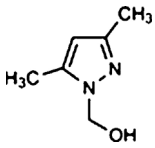
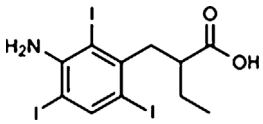
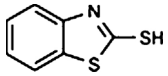
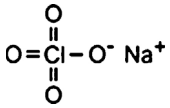
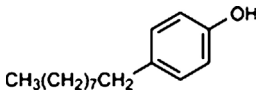
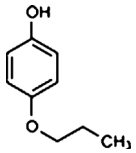
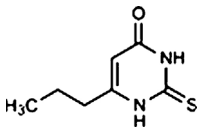
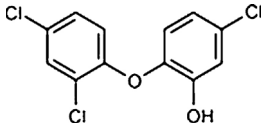
The amino acid sequences for human (*Homo sapiens*, Accession Number: AAA61217.2), porcine (*Sus scrofa*, Accession Number: P09933.1), and rat (*Rattus norvegicus*, Accession Number: EDM03234.1) TPO proteins were obtained from the NCBI Protein database (http://www.ncbi.nlm.nih.gov/protein/) and aligned using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/) (Goujon et al., 2010; Larkin et al., 2007). All three full-length peptide sequences were aligned together, and then for specific pair-wise comparisons of the six putative functional domains, i.e. human–porcine, human–rat, and rat–porcine. The 933-amino acid human TPO sequence was used as a guide for truncating the other two sequences and defining the functional domains. The six putative domains were defined as the Pro-myeloperoxidase (Pro-MPO) sequence (human amino acids 1–121), the MPO-like peroxidase region (human amino acids 121–741), the complement control protein repeat domain (CCP) (human amino acids 741–795), the epidermal growth factor receptor-like domain (EGF) (human amino acids 794–839), the transmembrane domain (human amino acids 846–870), and the intracellular domain (human amino acids 870–933) (Foti et al., 1990; Magnusson et al., 1987; McLachlan and Rapoport, 2007; Ruf and Carayon, 2006). Amino acid identity was determined for all six of these putative domains using ClustalW2 (Fig. 3 and Supplemental data 1).

3. Results

The guaiacol oxidation assay was successfully employed to evaluate all 12 chemicals in the training set for both rTPO and pTPO inhibition potential using thyroid microsomes. The presence of an effect in the rTPO and pTPO inhibition assays, the predicted IC_{50}, the lower and upper 95% confidence bounds, and the percent relative potency to MMI for each positive chemical are listed in Table 2. There was complete qualitative concordance for the positive and negative performance of all chemicals in the set between pTPO and rTPO; PTU, MBT, DPM, 4POP, and PERC were all positive across both species (Fig. 1), and DEHP, DEP, PNP, IOA, and TCS were negative across both species (Fig. 2).

The IC_{50} concentration values for the 6 positive chemicals varied between rTPO and pTPO (Figs. 1 and 2 and Table 2). MMI was more potent in the pTPO model, with an average $IC_{50} = 1.42$ μM compared to the rTPO average $IC_{50} = 2.22$ μM. The 95% confidence bounds on the IC_{50} for MMI with pTPO and rTPO overlap slightly, with an observed upper bound on the IC_{50} for pTPO (1.79 μM) and an observed lower bound for the IC_{50} values for rTPO (1.73 μM), suggesting a very small difference between the predicted experimental IC_{50} values for MMI across species. However, not all chemicals demonstrated greater potency (i.e. a lower IC_{50} value in the porcine model). DPM, 4POP, and PTU were all more potent in the rTPO model, and demonstrated lower IC_{50} values than in the pTPO model. While MMI demonstrated different potency across species, the best comparison of chemical potency is the percent relative potency to the positive control, MMI. Quantitative differences in the percent relative potency values were apparent across species, though there were few differences in the relative potency rank order between rTPO and pTPO (Table 2). The rank order, using relative potency, was as follows for rTPO inhibition: PTU > MMI > MBT > DPM > 4POP > PERC, and as follows for pTPO inhibition: MMI > PTU >MBT > DPM ≥ 4POP > PERC. PTU ($IC_{50,rat} = 1.28$ μM, 170% relative potency) more potently inhibited rTPO than MMI ($IC_{50,rat} = 2.22$), whereas PTU only had a 7.7% relative potency to MMI in the pTPO model. DPM also demonstrated a greater percent relative potency for rTPO despite similar rank

Table 1
12-Chemical training set description and source.

Chemical	Abbr.	CAS #	Source [a]	Purity [b]	Structure
Methimazole	MMI	60-56-0	S-A	≥99%	
Dibutylphthalate	DBP	84-74-2	S-A	≥99%	
Diethylhexylphthalate	DEHP	117-81-7	Fluka	≥97%	
Diethylphthalate	DEP	84-66-2	S-A	99.5%	
3,5-Dimethylpyrazole-1-methanol	DPM	85264-33-1	S-A	99%	
Iopanoic acid	IOA	96-83-3	TCI	>98%	
2-Mercaptobenzothiazole	MBT	149-30-4	Fluka	≥99%	
Sodium perchlorate	PERC	7601-89-0	S-A	≥98%	
4-*n*-Nonylphenol	PNP	104-40-5	L/AA[c]	95%/98%	
4-*n*-Propoxyphenol	4POP	18979-50-5	S-A	96%	
6-Propylthiouracil	PTU	51-52-5	S-A	99%	
Triclosan	TCS	3380-34-5	S-A	97%	

[a] *Sources*: S-A, Sigma–Aldrich, St. Louis, MO; Fluka, Fluka (Sigma–Aldrich), St. Louis, MO; L, Lancaster Synthesis Inc., Windham, NH; AA, AlphaAesar, Ward Hill, MA; TCI, TCI America, Portland, OR.
[b] Purity is as reported by the source.
[c] Two sources of 4-*n*-nonylphenol were used in the study, one for rTPO and one for pTPO studies.

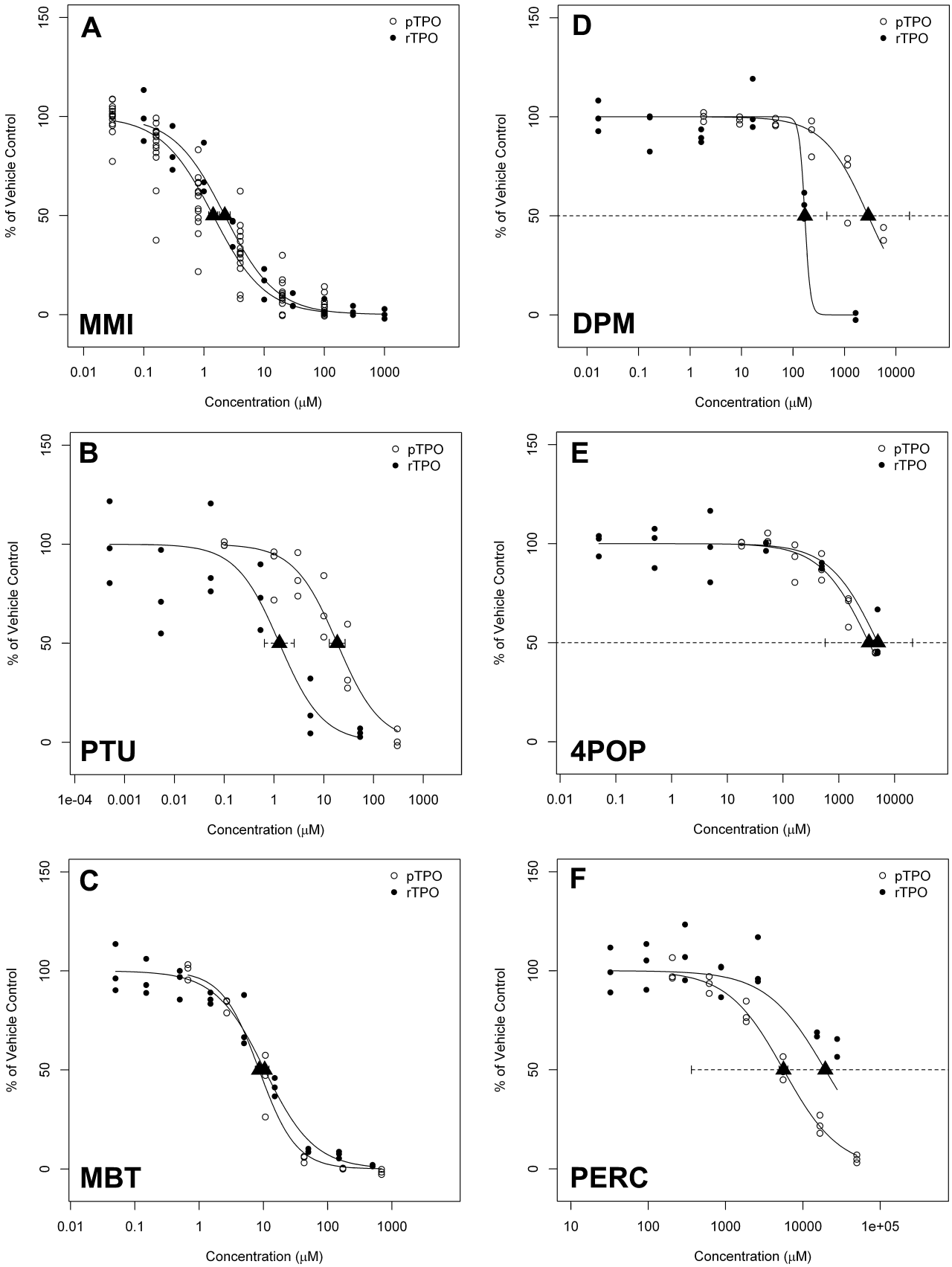


Fig. 1. Positive Chemicals with pTPO and rTPO in the TPO assay. Panels A, B, C, D, E, and F correspond to concentration-response data, as percent of vehicle control, for MMI, PTU, MBT, DPM, 4POP, and PERC, respectively. Open circles denote pTPO and closed circles denote rTPO. Curves indicate the four-parameter Hill fit for each chemical, by species. The IC_{50} for each curve is represented with a solid black triangle, and the SE around this value is shown with a dashed, bounded line.

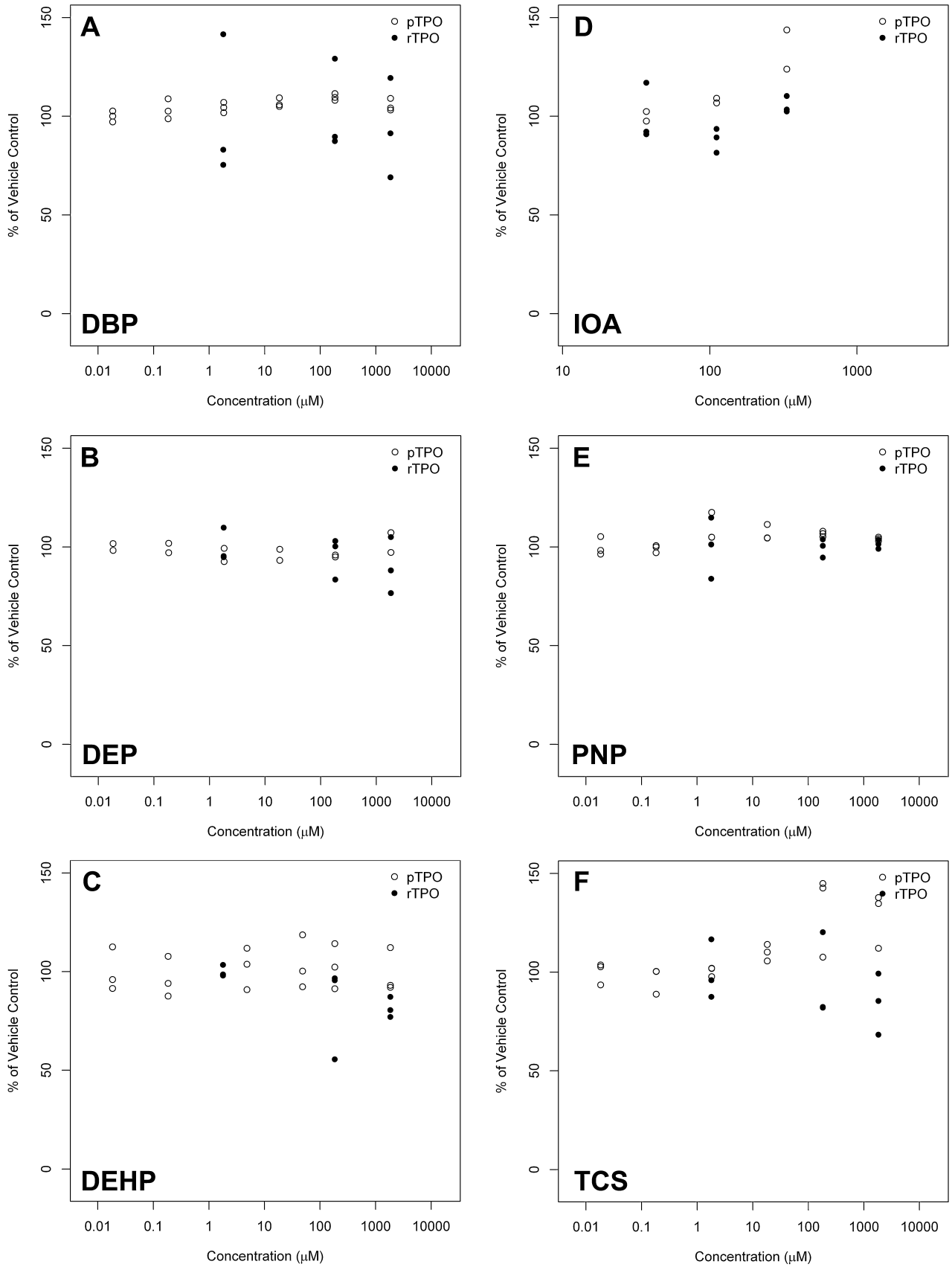


Fig. 2. Chemicals Tested Negative with pTPO and rTPO in the TPO assay. Panels A, B, C, D, E, and F correspond to concentration-response data, as percent of vehicle control, for DBP, DEP, DEHP, IOA, PNP, and TCS, respectively. Open circles denote pTPO and closed circles denote rTPO.

Table 2
Comparison of effects of the 12 chemical training set on pTPO and rTPO inhibition.

Chemical	pTPO IC_{50}[a] (μM)		rTPO IC_{50} (μM)		In-species relative potency to MMI (%)[b]	
	IC_{50}	LCB–UCB[c]	IC_{50}	LCB–UCB	pTPO	rTPO
MMI	1.42	1.12–1.79	2.22	1.73–2.84	100	100
PTU	18.4	12.3–27.9	1.28	0.521–3.21	7.7	170
MBT	8.56	7.05–10.3	10.4	8.36–12.9	17	21
DPM	2870	1930–4400	169	104–266	0.049	1.3
4POP	3490	2770–4430	4950	2720–unb[d]	0.041	0.045
PERC	5580	4890–6360	19,400	8070–unb[e]	0.025	0.011

[a] Chemical abbreviations: MMI, methimazole; PTU, 6-propylthiouracil; MBT, 2-mercaptobenzothiazole; DPM, 3,5-dimethylpyrazole-1-methanol; 4POP, 4-*n*-propoxyphenol; PERC, sodium perchlorate.
[b] The IC_{50} values were calculated using four-parameter Hill model fits with *R*.
[c] The percent in-species relative potency to MMI is calculated as the composite MMI activity for each species (pTPO or rTPO) divided by the activity of the test chemical, and multiplied by 100.
[d] Lower confidence bound (LCB) and upper confidence bound (UCB) are the lower and upper 95% confidence limits, respectively, around the predicted IC_{50} values.
[e] unb, upper 95% confidence limit is unbounded.

order across species; the percent relative potency of DPM for rTPO was approximately 27-times greater than the percent relative potency for pTPO. The potencies of 4POP and PERC were very low in the guaiacol assay for both rTPO and pTPO. 4POP demonstrated a relative potency that was nearly identical between rTPO and pTPO. Although the percent relative potencies for PTU and DPM were greater for rTPO than for pTPO, PERC acted as a far less potent inhibitor of rTPO than pTPO, with $IC_{50,rat}$ = 19,400 μM and $IC_{50,porcine}$ = 5580 μM, though the percent relative potency values are not dissimilar, largely owing to the extremely low potency of PERC. The IC_{50} values for MBT were extremely similar across species, 8.56 μM for pTPO and 10.4 μM for rTPO, and the species-difference in the MMI IC_{50} yielded similar percent relative potency values of 17 and 21% for pTPO and rTPO, respectively.

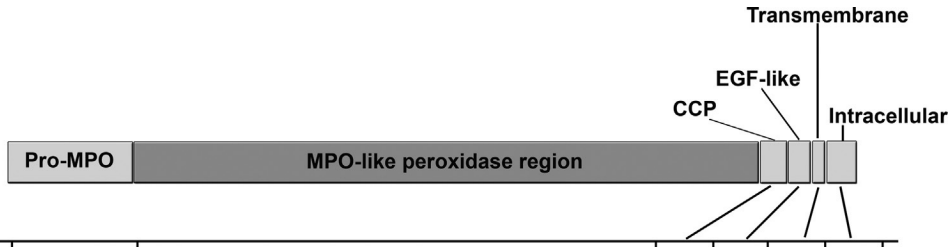
To better understand the cross-species agreement in TPO activity, the amino acid sequences for pTPO, rTPO, and human TPO (hTPO) were aligned (Fig. 3 and Supplemental data 1). There was a high degree of identity between full-length hTPO and pTPO, hTPO and rTPO, and rTPO and pTPO, 72%, 75%, and 67% respectively (Supplemental data 1). The six putative domains of hTPO were used to perform additional domain-specific comparisons across species, and these results are summarized in Fig. 3. The MPO-like peroxidase domain (amino acids 121–741) likely confers the peroxidase activity of the enzyme, and demonstrated the greatest interspecies identity of any putative functional domain at 75–80%. The intracellular domain, not thought to be involved in the catalytic function of TPO, demonstrated the lowest interspecies identity of 10–31%. In general, the hTPO to pTPO comparison and the hTPO to rTPO comparison demonstrated similar sequence identity across domains; the rTPO to pTPO comparison demonstrated a slightly lower sequence identity across domains.

4. Discussion

This study informs the applicability of a pTPO inhibition assay data for extrapolation of potential effects to *in vivo* rodent models via comparison of pTPO and rTPO inhibition using a 12-chemical training set in a guaiacol oxidation assay. The qualitative results from assays of the 12 test chemicals demonstrated close agreement across species for TPO inhibition. Importantly, this supports the use of pTPO data in planning *in vivo* rat studies, and suggests that the use of pTPO and/or rTPO assays and rat models of TH disruption are likely to be appropriate for informing human health hazard predictions. The minor quantitative differences in the predicted IC_{50} values for the 12 test chemicals may owe to different affinities of these compounds for the TPO enzyme across species and/or experimental variability. The 12-chemical set tested herein suggests that pTPO and rTPO behave very similarly when challenged by xenobiotics. This study is unique in its evaluation of multiple chemicals across structural classes in TPO inhibition assays. Previously, a comparison of microsomal rTPO and purified forms of pTPO, bovine lactoperoxidase, and human TPO activities in response to 10 μM genistein demonstrated 40–66% TPO activity decreases across species as indicated by quantification of MIT and DIT production (Doerge and Chang, 2002). However, limited available data on rTPO and pTPO comparisons underscore the importance of this work, and of the establishment of an understanding beyond a single test chemical.

The training set employed was developed to include chemicals that were previously identified as TPO inhibitors, as well as chemicals that were negative or possessed unknown activity. Four of the six positive chemicals for rTPO and pTPO inhibition, including MMI, PTU, MBT, and PERC, were consistent with previous literature reports of TPO inhibition, and the remaining two positive chemicals, DPM and 4POP, represented novel demonstrations of TPO inhibition *in vitro* by these chemical. DPM is a pyrazole, structurally similar to imidazoles except that pyrazoles have ortho-positioned nitrogen atoms instead of meta-positioned nitrogen atoms, and inhibited both pTPO and rTPO with low percent relative potency. This was a unique finding and is the first literature report of DPM-mediated TPO inhibition. However, DPM may be cytotoxic at high concentrations; DPM is cytotoxic at 500 μM in an amphibian thyroid explant culture system, but with no cytotoxicity at 165 μM (unpublished data; personal communication, Michael Hornung), approximately the IC_{50} for rTPO. An additional unique finding was detection of 4POP as a low potency TPO inhibitor; 4POP has tested positive for *in vitro* estrogenic activity (Schultz et al., 2002), but has no previously reported thyroid effects.

MMI and PTU, two confirmed positives in the training set, are structurally similar imidazole, anti-hyperthyroidism pharmaceuticals. MMI has been widely reported as a TPO inhibitor in both *in vitro* and *in vivo* models (Capen, 1994; Schmutzler et al., 2007a; Sugawara et al., 1999), and available clinical data suggest that the anti-hyperthyroid therapeutic action of MMI is to decrease TH synthesis *in vivo* via TPO inhibition (Cooper, 2005; Emiliano et al., 2010). PTU-mediated TPO inhibition confirms previous TPO-inhibition studies (Freyberger and Ahr, 2006; Sugawara et al., 1999; Vickers et al., 2012), as well as myriad studies of PTU-induced decreases in THs in rat models (Axelstad et al., 2008; Bakke et al., 1976; Gilbert and Sui, 2006; Hood et al., 1999b; Royland et al., 2008). However, PTU also inhibits peripheral deiodination of T4 to T3, and thus *in vivo* TH changes may be the result of multiple molecular-initiating events. The finding in this study that PTU was a more potent inhibitor of rTPO than MMI does not parallel previous findings in rats (Vickers et al., 2012). Recently MBT,



Human/Porcine	57%	80%	61%	67%	61%	29%
Human/Rat	52%	80%	63%	52%	63%	31%
Rat/Porcine	46%	75%	50%	54%	50%	10%

Fig. 3. Cross-species comparison of TPO domain identity. The six putative functional domains of the TPO enzyme are shown with the corresponding percent identity across species for each domain. The domains are based on the 933 amino acid human TPO protein.

a high production volume chemical intermediate for benzothiazole derivatives and rubber vulcanization accelerant, was identified as a pTPO inhibitor and thyrotoxicant using amphibian models (Tietge et al., 2012), and these findings were confirmed in this study. Finally, PERC was detected as a cross-species TPO inhibitor at extremely high concentrations, with IC_{50} values ranging from 6 to 17 mM. PERC primarily acts as a TH disruptor via competitive inhibition of iodide uptake by the sodium-iodide symporter (De Groef et al., 2006; Wolff, 1998), though this inhibition does not fully account for TH decreases observed in rats exposed to PERC (McLanahan et al., 2009). A previous report also identified potassium perchlorate as a low potency *in vitro* TPO inhibitor (Hosoya, 1963), and other reports provide conflicting evidence regarding potential PERC inhibition of MIT and DIT formation (McLanahan et al., 2009). The potential action of PERC on TPO activity is therefore somewhat unclear, though effects on TPO activity occurred at concentrations exceeding those at which PERC affects iodide uptake. Perchlorate decreased TH production at lower concentrations than for MMI *in vivo* in the *Xenopus laevis* tadpole assay (Tietge et al., 2010) and *in vitro* in the thyroid explant culture system (Hornung et al., 2010), suggesting that any effect on TPO inhibition would likely be a secondary mechanism to inhibition of iodide uptake for reduced T4 synthesis.

Six training set chemicals tested negative for TPO inhibition, including DBP, DEHP, DEP, IOA, PNP, and TCS. Only one of these, PNP, produced an unexpected negative result. PNP was previously reported to decrease *in vitro* hTPO activity (Schmutzler et al., 2007b), but was not detected as an rTPO or pTPO inhibitor in this study. This is in contrast to the other phenolic estrogen in the training set, 4POP, which tested positive as a low potency TPO inhibitor.

Three di-ester phthalates were included in the training set, DBP, DEP, and DEHP, as these chemicals have known endocrine effects, largely as anti-androgens, following enzymatic conversion to mono-ester phthalates (Christen et al., 2012; Howdeshell et al., 2008; Rider et al., 2010; Shen et al., 2009), but previously lacked the data needed to evaluate potential TPO inhibition. All of the tested phthalates produced negative results in the TPO inhibition assay. No TPO inhibition was observed for DBP, in accordance with a previous report that found no effects of DBP on TPO or NIS activity (Schmutzler et al., 2007b). The current work provides the first evidence that other phthalates including DEP and DEHP do not inhibit TPO. Phthalates may disrupt THs, as DBP and DEHP have previously demonstrated antagonism at the thyroid receptor (Shen et al., 2009), and DEHP is a known ligand for the human constitutive androstane receptor (CAR) (DeKeyser et al., 2009); activation of CAR along with other xenobiotic nuclear receptors may result in increased hepatic catabolism and excretion of THs to result in a systemic drop in serum TH concentration (Kretschmer and Baldwin, 2005; Smith et al., 2005; Sugatani et al., 2005; Zhou et al., 2005). DBP has also been reported to decrease sodium-iodide symporter expression *in vitro* (Breous et al., 2005). The training set data obtained here suggests that phthalate-induced TH disruption does not likely stem from TPO inhibition.

The other two negative training set chemicals were known thyroid-disrupting chemicals: TCS and IOA; however, neither is thought to act via TPO inhibition. IOA inhibits peripheral deiodinase I and conversion of T3 to T4 (Renko et al., 2012; Safer et al., 2009), and has been used in clinical and veterinary settings to treat hyperthyroidism (Earles et al., 2004; Gallagher and Panciera, 2009). TCS decreases TH in rats via induction of hepatic catabolic enzymes subsequent to a putative interaction with xenobiotic nuclear receptors (Paul et al., 2010a,b, 2012). Both of these chemicals tested negative for rTPO and pTPO inhibition.

The data do not demonstrate a consistent difference in sensitivity to TPO inhibitors for one species across the chemical test set. Though MMI demonstrated greater potency, i.e. a lower IC_{50} value, with pTPO, not all chemicals tested demonstrated greater potency with pTPO. In consideration of the percent relative potency values for the 12 chemical training set, the relative potency to MMI was greater for chemicals tested in the rTPO model, with the exception of PERC. Differences in the equipment used between laboratories and minor experimental differences prevent a direct comparison of test chemical IC_{50} values across species, and the percent relative potency to MMI represents the more appropriate comparison. The comparison presented herein suggests in general that the rTPO model may be more sensitive, i.e. lower concentrations of chemicals may inhibit rTPO, but this may be due to the decreased amount of total protein in each reaction when compared to the pTPO reactions. Testing more chemicals in both models would be necessary to develop a stronger statement regarding sensitivity to TPO inhibitors across species. However, it should be underscored that the results illustrate strong agreement of pTPO and rTPO qualitative response to TPO inhibiting chemicals, and provide similar quantitative results as well.

The high concordance of responses between rTPO and pTPO in the guaiacol oxidation assay is further reflected in the protein sequence alignment of rTPO and pTPO (Fig. 3). Though the amino acid sequence identity between full-length rTPO and pTPO (67%, Supplemental data 1) is high, the putative catalytic domain, the MPO-like peroxidase domain, demonstrates even greater sequence identity (75%). Domains with lower percent identity include the Pro-MPO domain, which is thought to be mostly removed from the mature hTPO protein during post-translational modification (McLachlan and Rapoport, 2007). The carboxy terminus of TPO is the least conserved portion of the protein. The juxtamembrane regions including the CCP and EGF-like domains, the

transmembrane domain, and the intracellular domain all demonstrate low sequence identity between rTPO and pTPO, but none of these domains confer TPO catalytic activity (Foti et al., 1990). As catalysis of TH production by TPO occurs on the lumenal side of the cell membrane in the thyroid follicular space, the poorly conserved intracellular domain likely has little influence on TPO activity. The strong sequence identity across species is observed for not only rTPO to pTPO, but also rTPO and pTPO to hTPO. The putative catalytic domain of hTPO demonstrated equivalent, high sequence identity to pTPO and rTPO (80%), suggesting that either TPO model would likely be appropriate for modeling an hTPO response.

Concordance across species for a set of chemicals was an important finding for future weight-of-evidence assessments of chemicals that may inhibit TPO. However, several uncertainties may limit the utility of using rat or porcine TPO for screening for human health. Rats and rat tissues may demonstrate an increased sensitivity to TPO inhibitors when compared to human thyroid tissues on the basis of gene expression changes (Vickers et al., 2012). Another unknown is whether chemicals that inhibit different TPO-catalyzed reactions would all test as positives in the guaiacol oxidation assay. The guaiacol oxidation assay serves as a surrogate for TPO-catalyzed coupling of iodotyrosine residues; however, TPO is a complex, multi-functional enzyme. TPO catalyzes both iodination of tyrosyl residues, via a two-electron oxidation of iodide, and coupling of iodotyrosyl residues, via a phenolic oxidation reaction, simultaneously (Doerge and Divi, 1995; Taurog et al., 1996); inhibition of TPO activity may proceed via interference with the coupling or iodination reactions, or suicide inactivation of the TPO enzyme itself (Divi and Doerge, 1994; Doerge and Chang, 2002; Doerge et al., 1994, 1998; Doerge and Decker, 1994; Doerge and Divi, 1995; Doerge and Niemczura, 1989; Freyberger and Ahr, 2006; Taurog et al., 1994). Inhibition of TPO activity in the guaiacol oxidation assay indicates inhibition of the one-electron phenolic oxidation, i.e. the coupling reaction; therefore, this assay may underestimate the potency of chemicals that may inhibit the iodide oxidation reaction. Guaiacol oxidation assays conducted without iodine, as performed in this study, favor the irreversible suicide inactivation of TPO over interference with the iodinating intermediate for chemicals such as the thiourea inhibitors, MMI and PTU (Freyberger and Ahr, 2006). Therefore, it is possible that the guaiacol oxidation assay results suggest a greater potency for MMI because more than one inhibition mechanism may be measured with this assay. The inhibition potency of MMI provides a point of reference to which the inhibition activity by other chemicals can be compared, acknowledging that multiple mechanisms of inhibition, i.e. competitive inhibition and suicide inactivation, may be present.

A further set of experimental uncertainties relates to interindividual differences in TPO activity and preparation of thyroid glands, as these individual differences and handling of the microsomes may result in different specific activities for TPO across sample preparations, potentially limiting quantitative comparisons between species or preparations of TPO. In addition, these animals were maintained on a soy-based diet, and soy isoflavones including genistein are known to inactivate TPO when measured *ex vivo*; however, these effects on TPO did not correspond to disruption of thyroid hormone homeostasis *in vivo* (Chang and Doerge, 2000). A soy-based diet was used in this study because this is the standard diet used in previous toxicology studies of thyroid disruption *in vivo*, and use of the guaiacol oxidation assay is intended to be predictive of these *in vivo* effects. It remains to be determined whether the use of microsomes from animals fed a soy-based diet impacts the ability to resolve TPO inhibitors or changes the relative potencies with the guaiacol oxidation assay.

Here we report a qualitative concordance of pTPO and rTPO inhibition for all 12 chemicals in an assay training set. Further, the rank order of relative potency to a positive control, MMI, was largely consistent across species. Together, these data support the use of either pTPO or rTPO data to detect the effects of chemicals on TPO. Future work should involve correlation of *in vivo* measurements of THs in rat with *in vitro* measurements of TPO inhibition to understand the degree of adversity potentially imposed by TPO inhibition, and to link TPO inhibition with an adverse outcome pathway for TH disruption at the individual level (Ankley et al., 2010; Zoeller et al., 2007b). This understanding could drastically increase the utility of TPO inhibition data for prioritization of thyroid-disrupting chemicals.

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tox.2013.08.006.

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